Table of Contents

Materials. All Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified and were used as received. DNA oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). AmpliScribe™ T7-Flash™ Transcription Kit and CircLigase II ssDNA Ligase was purchased from Epicenter (Madison, WI, USA). Bst 2.0 DNA polymerase and PvuI were purchased from New England BioLabs Inc. (Ipswich, MA, USA). Plasmids pCAG-T3-hCAS-pA encoding the Cas9 protein (Addgene No. 48625) and pCAG-GFP encoding EGFP (Addgene No. 11150) were purchased from Addgene (Cambridge, MA, USA). Linear polyethyleneimine (PEI) "max" (M.W. 40,000) was purchased from Polysciences Inc. (Warrington, PA, USA).

Clone, expression and purification of Cas9 protein. The human codon optimized *S. pyogenes* Cas9 gene with two nuclear localization sequences (NLS) at the N- and C- termini (Addgene 48625)[1] was amplified and sub-cloned into pET-28a vector (Novagen) with primers Cas9-F/Cas9-R (Table S1), adding a N-terminal His_6-tag to the expressed Cas9. *E. coli* Rosetta (DE3) pLysS cells was transformed with pET28a-Cas9 and cultured for Cas9 expression. Briefly, a fresh *E. coli* colony was inoculated into 5 mL LB medium (supplemented with 10 μg/mL kanamycin and 34 μg/mL chloromycetin) and cultured at 37 °C overnight. The cell culture was then diluted with fresh LB medium by 100-fold and continued to culture for another 2-3 h until the OD_{600} reached 0.6-0.8. 0.5 mM isopropyl β-D-1-thiogalactopy- ranoside (IPTG) was added

to induce Cas9 expression at 20 °C for 8 h. The cells were then collected by centrifugation (4000 ×g, 15 min), suspended in Buffer A (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 10 mM imidazole and 1 mM PMSF) and lysed by sonication. Cell debris was removed by centrifugation (20000×g, 20 min) and the clear lysate was added to a column containing 1 mL Ni-NTA resin (Qiagen). After washing the column with Buffer B (20 mM Tris-HCl pH 8.0, 0.5 M NaCl and 60 mM imidazole), Cas9 was eluted with Buffer C (20 mM Tris-HCl pH 8.0, 0.5 M NaCl and 500 mM imidazole) and dialyzed against Buffer D (20 mM HEPES pH 7.4, 150 mM KCl, 1 mM DTT and 10% glycerol) at 4 °C overnight. The purified Cas9 was quantified by Bradford assay (Bio-Rad) and analyzed by SDS-PAGE.

Transcription and purification of single-guide RNA (gRNA). The sgRNA was transcribed *in vitro* with AmpliScribe™ T7-Flash™ Transcription Kit (Epicentre) according to manufacturer's instructions. Transcription templates encoding a T7 promoter followed by the sgRNA were synthesized by IDT with the sgRNA containing a 20 bp EGFP targeting sequence and a control guiding RNA (cgRNA) that does not target EGFP or any genes in human genome (Table S1). The transcribed RNA was extracted by phenol:chloroform:IAA (Ambion) with Phase Lock Gel (5 Prime) to separate the RNA containing water phase. After removing unincorporated nucleotide with illustra Microspin G-50 columns (GE Healthcare), the RNA was ethanol precipitated and re-suspended in DEPC treated water. Purified RNA was analyzed by agarose gel electrophoresis and quantified with Nanodrop 2000c (Thermo Scientific).

Plasmid DNA cleavage assay to detect Cas9 activity. Plasmid pCAG-GFP containing the EGFP gene (Addgene 11150)[2] was linearized with PvuI (NEB), purified by GeneJET Gel Extraction Kit (Thermal Scientific) and used as the substrate for Cas9 activity assay. In a reaction volume of 20 μl containing NEBuffer 3 and linearized plasmid (300 ng), purified Cas9 (50 nM) and sgRNA (50 nM) were added. After digestion for 1 h at 37 °C, the DNA was analyzed by 0.8% agarose gel electrophoresis.

Preparation and characterization of DNA NC. The DNA NCs were prepared by RCA using cyclized single stranded DNA (ssDNA) templates. 5' phosphorylated linear ssDNA templates (Table S1) were cyclized by CircLigase II ssDNA ligase (Epicentre) following manufacture's instructions. Unligated ssDNA chains were removed with 1U Exo I (NEB) at 37°C for 45 min followed by heat inactivation at 80°C for 15 min. The cyclized ssDNA template (10 pmol) was added into 1 mL 1× isothermal amplification buffer (NEB) together with 0.5 μM primer and 200 μM dNTP and heated to 95°C for 5 min. After hybridizing the template and primer by cooling the mixture to room temperature, Bst 2.0 DNA polymerase (0.2 U/μL) was added to initiate the RCA. The RCA was performed at 65°C overnight and the denatured polymerase after the reaction was removed by centrifugation at 14,000×g for 2 min. The supernatant was recovered and dialyzed against DI water using a Slide-A-Lyzer (20K MWCO, Thermo Scientific) for 48 h. The synthesized DNA NCs were analyzed by 0.8% agarose gel electrophoresis

and .Nanodrop 2000C (Thermal Scientific) was applied to measure the concentration and purity of the DNA NC. NC with high purity ($A_{260}/A_{280} > 1.8$) was used for further studies. To evaluate the stability of NC, 300 ng NC was incubated with Cas9 (50 nM) and gRNA (50 nM) in NEBuffer 3 at 37°C for 24 h and then analyzed using 0.8% agarose gel electrophoresis. Zeta potential and particle size of NC were measured with a Zetasizer (Malvern). To image the NC by atomic force microscopy (AFM), the NC was dropped and dried onto a silicon wafer (Ted Pella) and analyzed on a Nanoscope AFM instrument (Veeco, Santa Barbara, CA) using tapping mode in ambient air.

Assembly and characterization of Cas9/sgRNA/NC/PEI. Purified Cas9 and sgRNA at various molar ratios (4:1 – 0.5:1) were mixed in PBS and incubated at room temperature for 5 min, followed by the addition of DNA NC (NC:sgRNA weight ratio of 4:1) and incubated at room temperature for another 5 min. Afterwards, PEI "max" (Polysciences) was coated onto Cas9/sgRNA/NC at PEI:sgRNA weight ratio of 5:1 and equilibrated at room temperature for 5 min. The assemblies were further diluted to the concentration of sgRNA at 100 nM in deionized water for particle characterization or Opti-MEM medium (Life Technologies) for cell study. Size and zeta potential of Cas9/gRNA/NC/PEI were analyzed by a Zetasizer (Nano ZS, Malvern). AFM imaging was performed using a Nanoscope (Veeco, Santa Barbara, CA) on silicon wafer (Ted Pella) as described above. For TEM imaging, the Cas9/sgRNA/NC/PEI was dropped onto a

TEM copper grid (300 mesh, Ted Pella) and stained with 2% uranyl acetate (w/v, in 50% ethanol). TEM images were observed on a JEM-2000FX (Hitachi) at 200 kV. The assembly was also visualized with confocal laser scanning microscope (CLSM, LSM 710, Zeiss) to confirm the colocalization of the components. Cas9 was conjugated with Alexa Fluor 647 C2 maleimide (AF647), PEI was conjugated with FITC NHS ester (Life Technologies) and the NC was stained with Hoechst 33342, a nucleic acid dye that stains only DNA but not RNA.[3]

Cell culture and EGFP gene disruption assay. The reporter cell line U2OS.EGFP with a single copy of destabilized EGFP gene integrated into the genome was a generous gift from Dr. J Keith Joung at Massachusetts General Hospital.[4] The cells were cultured in a 37 °C incubator under 5% CO_2 and 90% humidity with full serum medium: Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) FBS, 2 mM GlutaMAX (Life Technologies), penicillin (100 U/mL) and streptomycin (100 μg/mL). U2OS.EGFP cells were seeded into 24-well plates (~25,000 cells per well) one day before the transfection. When the cells reached 70% confluence, the medium was replaced with 0.5 mL Opti-MEM medium (Life Technologies) containing the Cas9/gRNA loaded nanoparticles (gRNA concentration at 100 nM). After incubation for 4h, the Cas9 containing medium was replaced with fresh full serum medium. Two days after the delivery, the cells were analyzed using a fluorescent microscope (IX71, Olympus). For the flow cytometry analysis, the cells were washed with ice cold PBS twice and trypsinized with 0.05 %

trypsin (Mediatech) at 37 °C for 1-2 min. Afterwards, the cells were washed and resuspended in full serum medium and analyzed by a BD Accuri C6 flow cytometer (BD Biosciences).

SURVEYOR assay to detect genomic modifications. Genomic DNA of U2OS.EGFP cells was harvested 2 d after the delivery using GeneJET Genomic DNA Purification Kit (Thermo Scientific) according to manufacturer's instructions. The gRNA targeted genomic locus was amplified with Phusion Hot Start II High Fidelity DNA Polymerase (NEB) using primers T7EI-F/T7EI-R (Table S1). Touchdown PCR program ((98°C for 10 s; 66-56°C with -1 °C/cycle for 15s, 72°C for 60 s) for 10 cycles and (98°C for 10 s, 56°C for 15 s, 72 °C for 60 s) for 25 cycles) was used to reduce non-specific amplifications. The amplicons were then purified by gel extraction and 200 ng of the purified DNA was added to 20- μL cleavage reaction containing 1× NEBuffer 2. After heating to 95°C for 5 min, the mixture was cooled to form heteroduplex DNA. Afterwards, 1 μL T7EI (10 U/μl, NEB) was added and incubated at 7 °C for 15 min. The digested DNA was analyzed using 2% agarose gel electrophoresis. Indel formation efficiencies were calculated using Image J.

DNA sequencing to detect genomic mutations. Purified PCR amplicons of the T7EI assay were cloned into Zero Blunt TOPO DNA sequencing vectors (Life Technologies). The cloned

plasmids were purified by GeneJET Plasmid Miniprep Kit (Thermo Scientific) and sequenced by Eton Bioscience Inc. (RTP, NC, USA) with T7 promoter primer.

Determination of endocytosis pathways. Cas9 was fluorescently labeled with AF647 to track its uptake. U2OS.EGFP cells were seeded in 24-well plates (~25000 cells/well) and cultured for 2 d. Then the cells were pre-incubated with several endocytosis inhibitors[5], such as chlorpromazine (CPZ, 10 μM) for clathrin-mediated endocytosis, nystatin (NYS, 25 μg/mL) for caveolin-mediated endocytosis, methyl-β-cyclodextrin (MCD, 3 mM) for lipid raft and amiloride (AMI, 1 mM) for macropinocytosis, for 1h at 37 °C, respectively. Afterwards, the cells were incubated with AF647-Cas9/sgRNA/NC/PEI for another 2 h in the presence of the inhibitors. Cells were then washed, trypsinized and resuspended in full serum medium, intracellular AF647 fluorescence intensities were analyzed by flow cytometry.

Intracellular distribution of Cas9. U2OS.EGFP cells were seeded in confocal dishes (MatTek) at a density of 1×10^5 per well and cultured for 24 h. To image the nuclear accumulation of Cas9, the cells were incubated with AF647-Cas9/sgRNA/NC/PEI for 1 h, 2 h, 4h and 6 h. After washing with 4 °C PBS twice, the cells were stained with Hoechst 33342 (1 μg/mL) for 10 min. Washed with cold PBS twice again, the cells were observed with CLSM immediately.

***In vitro* cytotoxicity.** U2OS.EGFP cells delivered with Cas9 were analyzed for cell survival using flow cytometry with TO-PRO-3 live/dead cell stain (Life Technologies).[6] Briefly, the U2OS.EGFP cells were seeded in 24-well plates (~25000 cells/well) and cultured for 24 h. Then the cells were exposed to Cas9/sgRNA/NC-12/PEI and Cas9/sgRNA/PEI at different Cas9 concentrations for 4 h. Afterwards, the cells were washed with PBS and stained with TO-PRO-3 live/dead cell stain (1 μ M) for 15 min. Washed, trypsinized and resuspended, the cells were then analyzed by flow cytometry.

***In vivo* EGFP disruption.** All animal experiments were conducted according to the Guide for Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committee (IACUC) of University of North Carolina at Chapel Hill and North Carolina State University. To set up the U2OS.EGFP tumor model, the female nude mice (6 weeks, J:NU, The Jackson Laboratory) were subcutaneously inoculated with 1×10^7 U2OS.EGFP cells. One mouse was inoculated with one tumor and when the volume of the tumors reached 200 - 400 mm^3, the mice were intratumorally injected with 50 μL of the ~ 56 nm nanoparticles (Cas9/sgRNA/NC-12/PEI or Cas9/cgRNA/NC-12/PEI) in PBS (Cas9 concentration at 5 μM). At day 10, the mice were euthanized and the tumors were collected, washed by saline thrice and followed by fixation in the 10% neutral buffered formalin. Tumor tissues within 5 mm of distance from the point of injections were sectioned. Cas9-mediated EGFP disruptions were visualized by staining the tumor sections with FITC conjugated GFP antibody (Thermo

Scientific) and the nuclei were counterstained with Hoechst 33342. The stained slides were observed with CLSM.

Statistics. All results were presented as Mean ± SD Statistical analysis was performed using two-tailed student's *t-test*. The difference between experimental groups and control groups were considered statistically significant when $p < 0.05$ or highly significant when $p < 0.01$.

Table S1. Sequences of DNA oligos

		Sequences	Notes
Cas9 Clone Primer	Cas9-F	5'-GCAAATGGGTCGCGGATCCCCAAAGAAGAAGCGG-3'	BamH
	Cas9-R	5'-CGAGTGCGGCCGCAAGCTTTCACACCTTCCTC-3'	HindIII
RNA Transcription Template	sgRNA	5'-**GTTTTTTTTAATACGACTCACTATAGG**GCACGGGCAGCTTGCCGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT-3'	**T7 Promoter** GFP Targeting Non-Targeting Sequence
	cgRNA	5'-**GTTTTTTTTAATACGACTCACTATAGG**GTAACCGTGCGGTCGTACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT-3'	
RCA Template	NC-23	5'PO_4-GGGCACGGGCAGCTTGCCGGTGGAAGCTAGATGCATCTAGCAAGCGCCGCCACTGATTTCACCGCTTCAAGCTAGATGCATCTAGCAAT-3'	gRNA Binding PAM Palindromic Sequence NC Primer Binding
	NC-12	5'PO_4-GCTACCGGGCAGCTTGCATCAATAAGCTAGATGCATCTAGCAAGCGCCGCCACTGATTTCACCGCTTCAAGCTAGATGCATCTAGCAAT-3'	
	NC-0	5'PO_4-GAGAAACGAGTGCGGTCACAGCTAAGCTAGATGCATCTAGCAAGCGCCGCCACTGATTTCACCGCTTCAAGCTAGATGCATCTAGCAAT-3'	
RCA Primer	NC-F	5'-GTGGCGGCGC-3'	
SURVEYOR assay primer	T7EI-F	5'-GGAGTTCCGCGTTACATAACTTACG-3'	
	T7EI-R	5'-AACCTCGACTAAACACATGTAAAGCATG-3'	

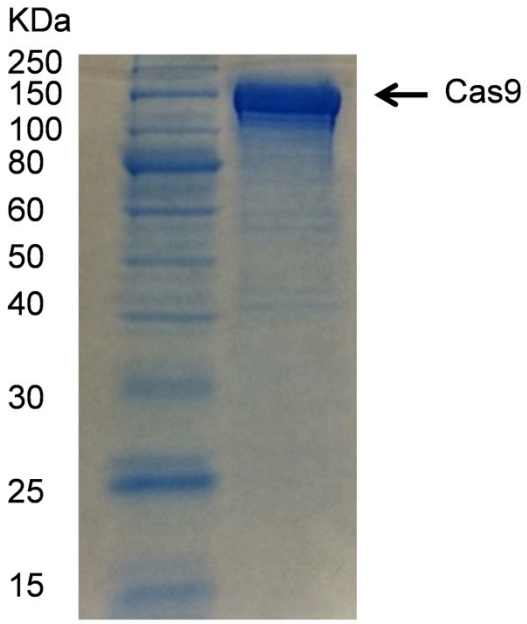

Figure S1. SDS-PAGE (12%) of purified Cas9. The purified Cas9 showed molecular weight of ~160 KDa.

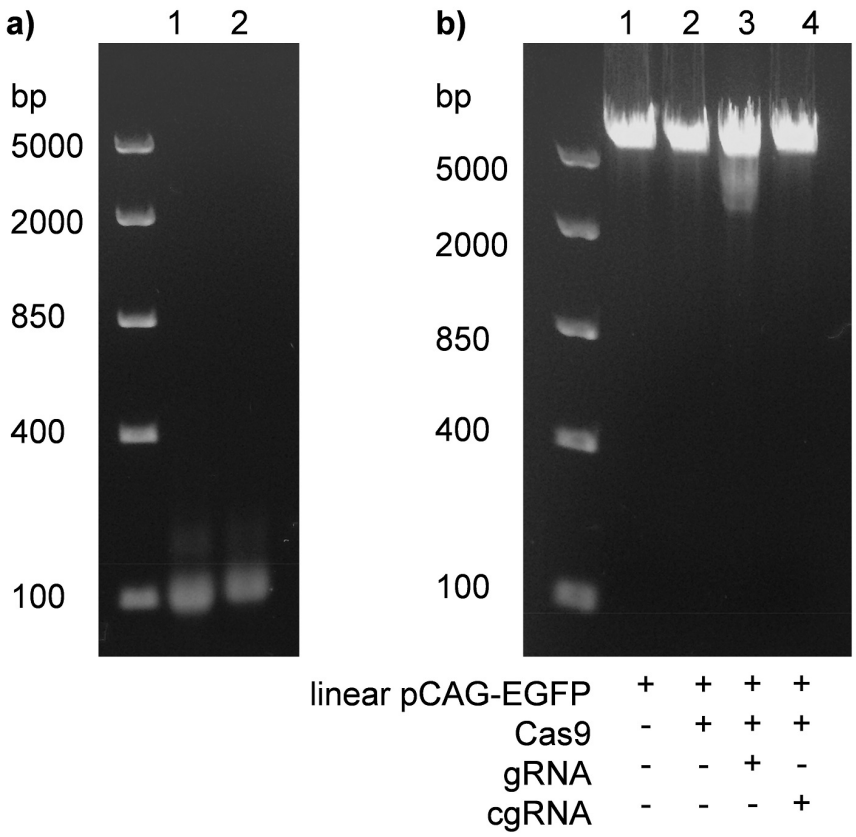

Figure S2. a) Agarose gel electrophoresis (0.8%) of purified sgRNA (lane 1) and cgRNA (lane 2). b) Cas9 activity assay using linearized plasmid pCAG-EGFP (5556 bp) as substrate. Only Cas9 complexed with sgRNA can digest the plasmid DNA.

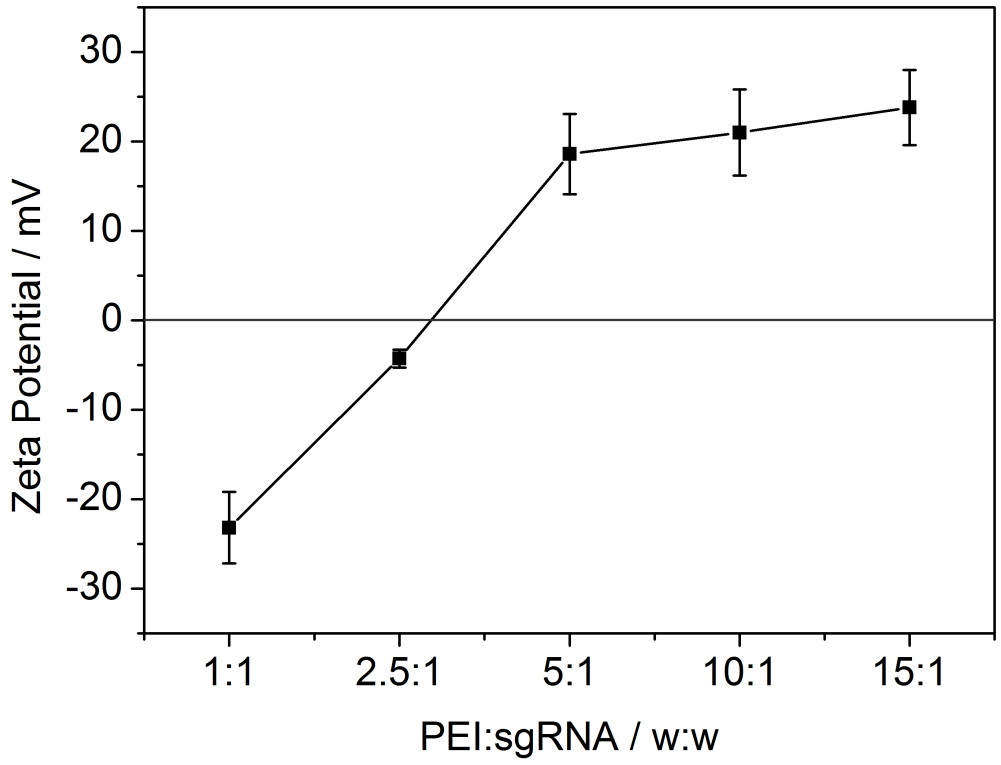

Figure S3. Optimization of PEI concentration for coating Cas9/sgRNA/NC-12 by measuring the zeta potential.

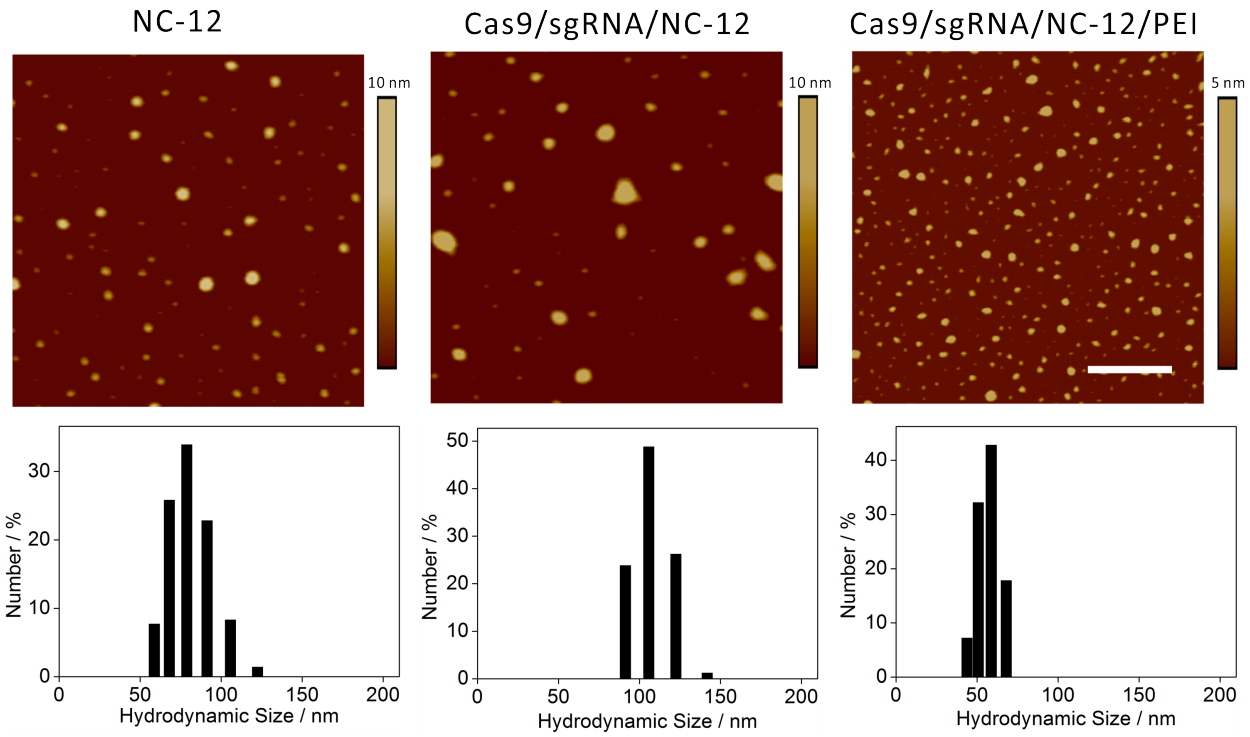

Figure S4. Hydrodynamic size distributions and AFM images of NC-12, Cas9/sgRNA/NC-12 and Cas9/sgRNA/NC-12/PEI. Scale bar 400 nm.

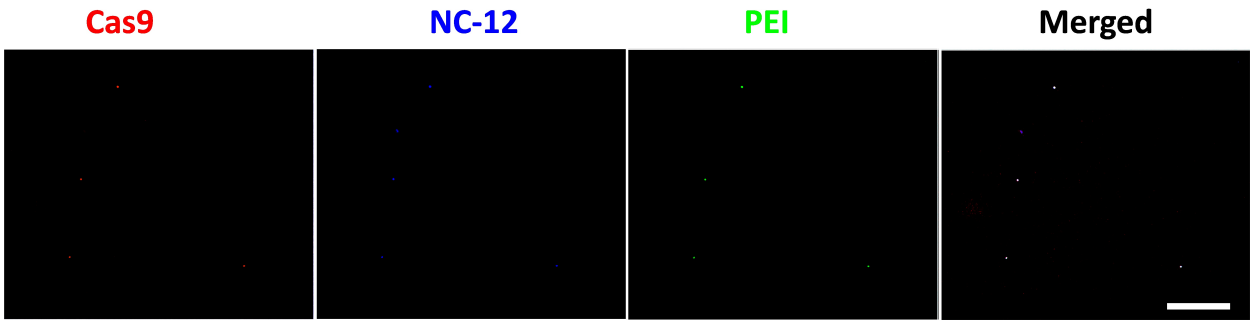

Figure S5. CLSM images of Cas9/sgRNA/NC-12/PEI assembly. Red for Cas9 stained with AF647, blue for NC-12 stained with Hoechst 33342 and green for PEI labeled with FITC. Scale bar is 20 μm.

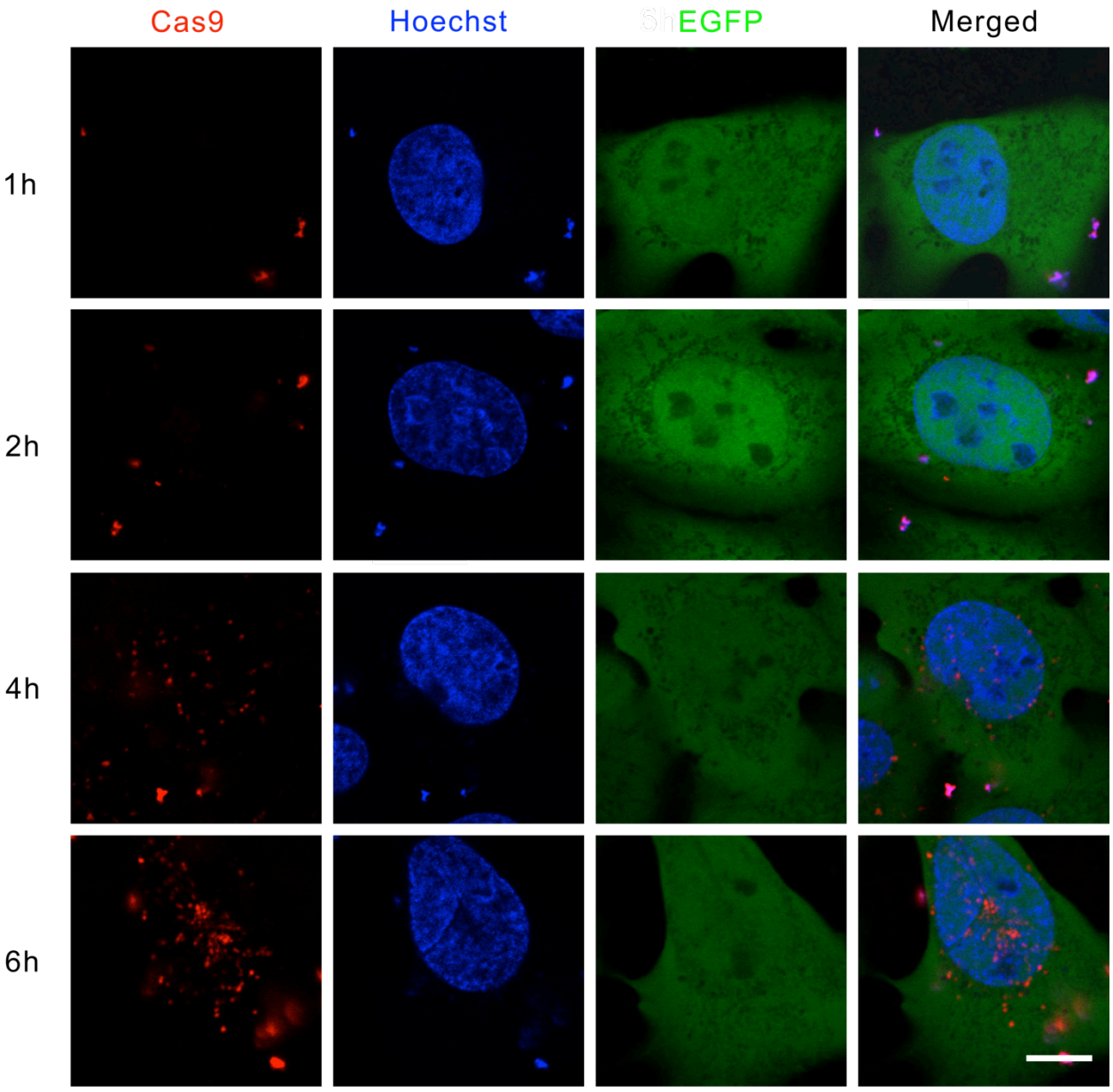

Figure S6. Confocal laser scanning microscopy images of U2OS.EGFP cells incubated with Cas9/sgRNA/NC-12/PEI for 1 h, 2 h, 4 h and 6 h (Cas9 and sgRNA concentrations at 100 nM).

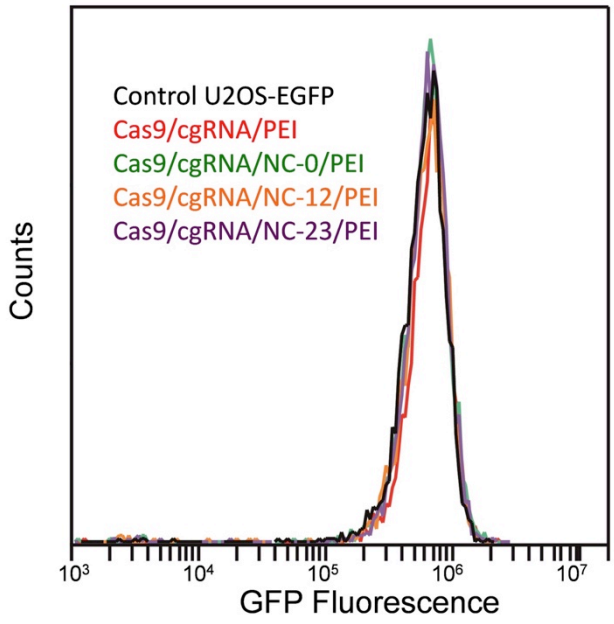

Figure S7. Flow cytometry analysis of U2OS.EGFP cells treated with formulations containing cgRNA, which did not show any EGFP disruption efficacy.

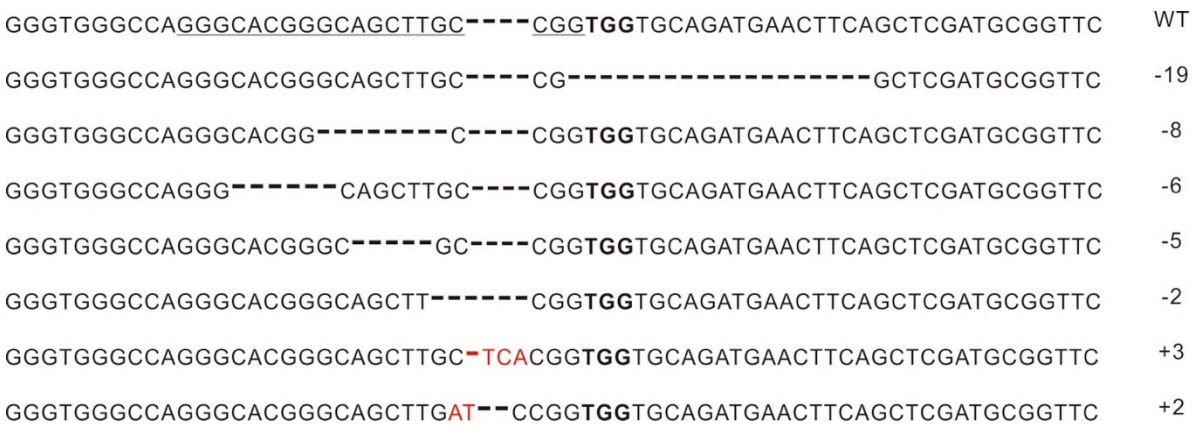

Figure S8. DNA sequencing of Cas9/sgRNA targeted gemonic locus in U2OS.EGFP cells. Target sequence complementary to the sgRNA is underlined and PAM sequence is shown in bold. Mutations were detected in 7 out of 20 sequenced clones. Number of insertion/deletion as compared to the wild type sequence is shown on the right.

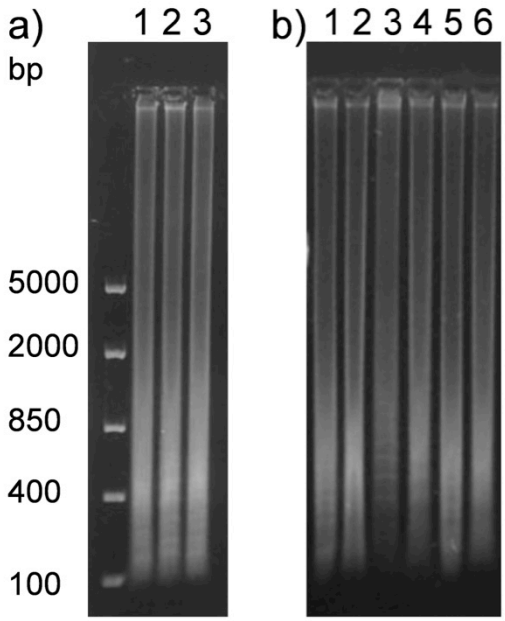

Figure S9. a) Agarose gel electrophoresis (0.8%) of synthesized NC-23, NC-12 and NC-0 in lane 1, 2 and 3, respectively. b) Analysis of NC stability after incubating with Cas9/sgRNA for 24 h. Lane 1, 3, 5 were for untreated NC-23, NC-12 and NC-0 and lane 2, 4, 6 showed Cas9/sgRNA treated NC-23, NC-12 and NC-0, respectively.

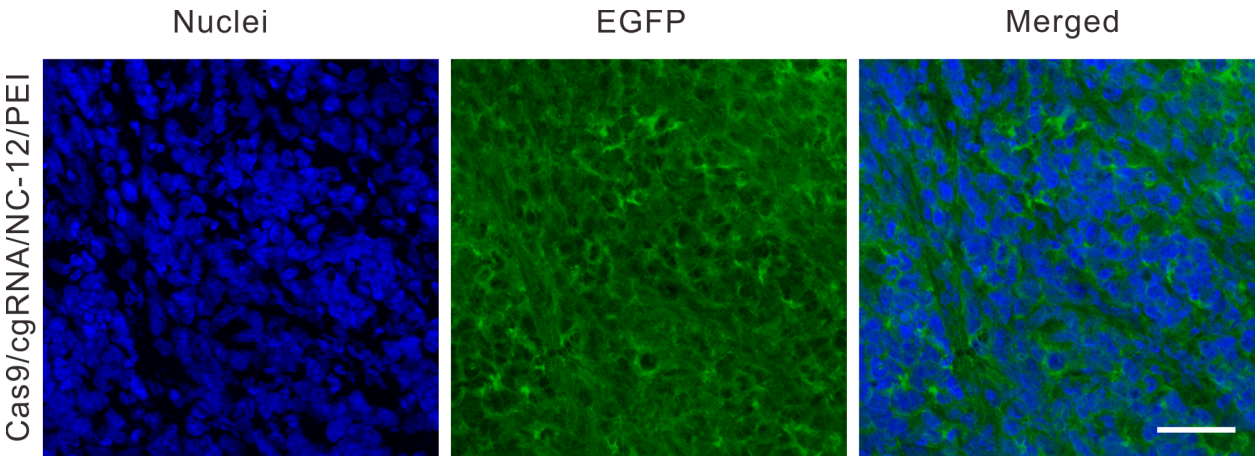

Figure S10. Tissue section of tumor treated with Cas9/cgRNA/NC-12/PEI. The EGFP was stained by FITC conjugated GFP antibody and nuclei were stained with Hoechst 33342. The Scale bar is 50 μm.

References

[1] W. Fujii, K. Kawasaki, K. Sugiura, K. Naito, *Nucleic Acids Res.* **2013**, *41*, e187.

[2] T. Matsuda, C. L. Cepko, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 16-22.

[3] H. Crissman, Z. Darzynkiewicz, R. Tobey, J. Steinkamp, *Science* **1985**, *228*, 1321-1324.

[4] Y. Fu, J. A. Foden, C. Khayter, M. L. Maeder, D. Reyon, J. K. Joung, J. D. Sander, *Nat. Biotechnol.* **2013**, *31*, 822-826.

[5] R. Mo, T. Jiang, R. DiSanto, W. Tai, Z. Gu, *Nat. Commun.* **2014**, *5*, 3364.

[6] J. A. Zuris, D. B. Thompson, Y. Shu, J. P. Guilinger, J. L. Bessen, J. H. Hu, M. L. Maeder, J. K. Joung, Z.-Y. Chen, D. R. Liu, *Nat. Biotechnol.* **2015**, *33*, 73-80.